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Signaling and Prostate Cancer

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## Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	6
Reportable Outcomes.....	6
Conclusions.....	7
References.....	7
Appendices.....	8

## Introduction

Most prostate cancer patients respond initially to androgen ablation and antiandrogen therapy. However, virtually all patients will relapse due to acquisition of the growth of the androgen-independent tumor cells. The molecular mechanism characterizing prostate cancer progression from androgen-dependence to androgen-independence is incompletely understood. We propose that Signal Transducers and Activators of Transcription 3 (Stat3) both regulates the expression of Stat3 target genes, and interacts with AR in prostate cancer cells. The experiments proposed in this application are based upon the hypothesis that Stat3 activation alters androgen receptor signaling pathways, that in turn results in the loss of growth control in prostate cancer cells. We propose to determine the consequence of Stat3 activation in prostate cancer cell growth and to determine the molecular basis of Stat3 interactions with androgen receptor signaling.

## Body

Since the approval of this application, we have made significant progress of task 1 (i.e., to examine the role of Stat3 activation in prostate cancer cells (months 1-18).

Task 1A. To establish a series of prostate cancer cell lines demonstrating constitutive Stat3 activation (months 1-6). We have established Stat3 constitutively activated cell sublines in LNCaP cells. These cells express activated Stat3 as examined by EMSA using Stat3 consensus binding sequences (reference 1).

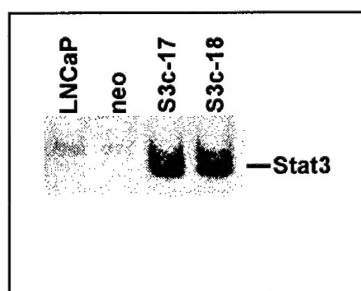
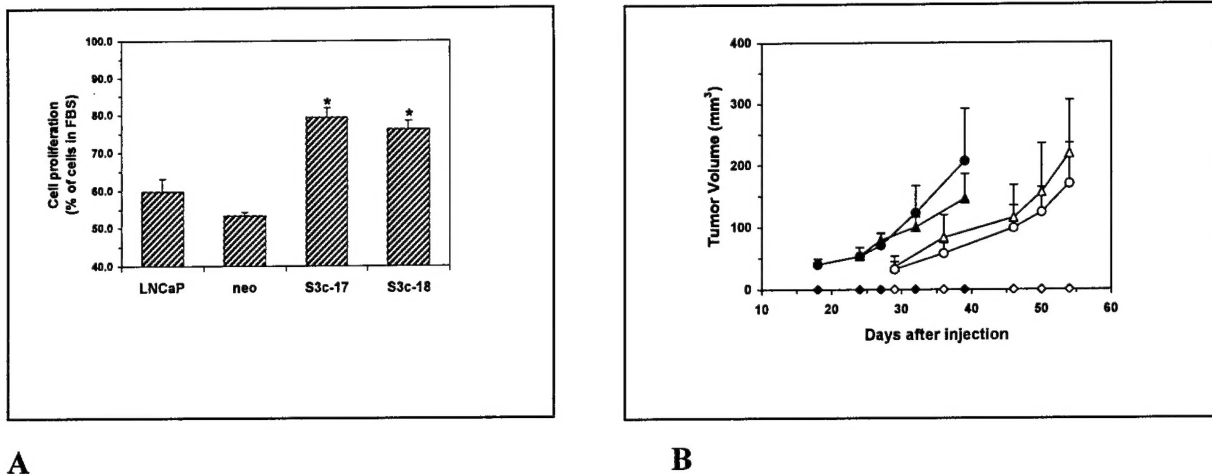


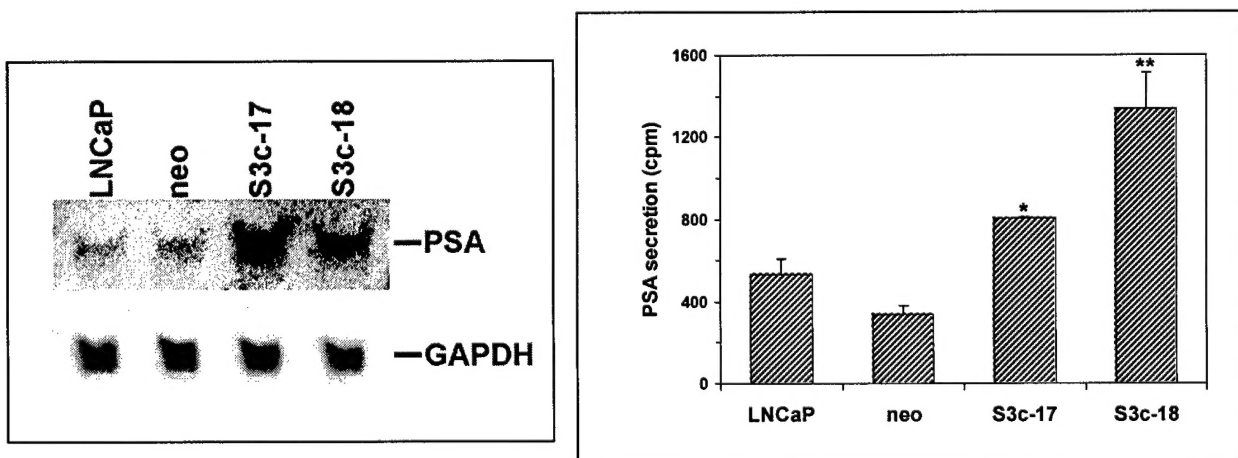
Fig 1. Stat3 DNA-binding activity in LNCaP and LN series cell lines. Whole cell extracts (20  $\mu$ g) were subjected to EMSA using a  $^{32}$ P-labeled oligonucleotide probe containing the consensus binding motif for Stat3.

Task 1B. To examine the effect of Stat3 activation on these cell growth in vitro and in vivo (months 7-12). We demonstrated that cells expressing constitutively activated Stat3 can enhance LNCaP androgen independent growth in vitro as analyzed by MTT assay (Fig 2A) and LNCaP growth in the castrated nude mice (Fig 2B), suggesting that Stat3 can enhance androgen independent growth of androgen-dependent LNCaP cells (reference 1).

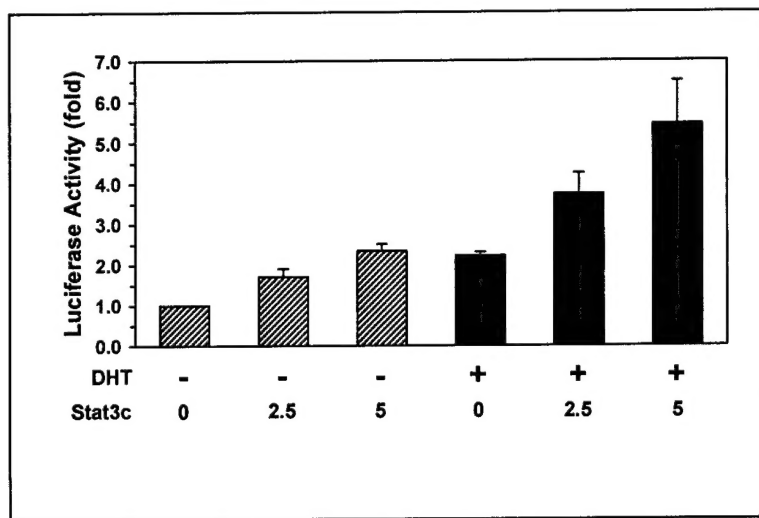


**Figure 2.** Stat3 enhances androgen-independent growth *in vitro*. **(A)** Effect of Stat3 on LNCaP cell growth in the presence and absence of androgen *in vitro*. Cells were cultured in RPMI-1640 supplemented with either 10% FBS or 10% charcoal-stripped FBS. Cell proliferation values in charcoal-stripped FBS were expressed as % relative to the complete FBS. \*,  $P < 0.05$ . **(B)** Stat3 induces androgen-independent growth *in vivo*. Tumor growth curve in the intact and castrated male nude mice. Parental LNCaP cells and neo clone ( $\diamond$ ) or clones that overexpress activated Stat3 (S3c-17,  $\nabla$ ; S3c-18,  $\circ$ ) were injected into the intact (filled symbols) or castrated (open symbols) male nude mice ( $n = 10$  for each condition).

Task 1C. To examine the effect of Stat3 activation on the expression of Stat3 target genes and AR-mediated genes (months 7-18). Prostate specific antigen (PSA) is a typical AR-mediated gene. We demonstrated that Stat3 can enhance PSA express both in mRNA levels as examined by Northern blot (Fig 3A) and in protein levels as examined by ELISA (Fig 3B). In addition, Stat3 can enhance PSA promoter activity and ARE-containing gene transactivation (Fig 4 and reference 1).



**Figure 3.** Stat3 enhances PSA expression. (A) PSA mRNA expression in Stat3 overexpressing clones (S3c-17, S3c-18), vector control (neo), and LNCaP cells examined by Northern blot analysis using 20 µg of total RNA. GAPDH is a control for equal loading. (B) PSA protein secretion in the absence of androgen. PSA secretion was quantitated by PSA immunoradiometric assay of 50 µl of supernatant of cell culture in phenol red-free RPMI containing 10% charcoal-stripped serum. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .



**Figure 4.** (A) Effect of Stat3 on PSA promoter activity in the absence of DHT and in the presence of 10 nM of DHT. LNCaP cells were transiently transfected with PSA-luc reporter, and increasing doses (0, 2.5, 5 µg) of Stat3 expression plasmid. Total DNA content was kept constant in all wells. HeLa cells transiently transfected with or without AR expression plasmid, PSA-luc reporter, and increasing doses (0, 2.5, 5 µg) of Stat3c expression plasmid in the presence of 10 nM of DHT. Total DNA content was kept constant in all wells. The luciferase activity was measured. Results are displayed as the average of four independent experiments. RLU: relative light units.

#### Key research accomplishments

- We demonstrated that Stat3 plays a critical role in prostate cancer growth.
- Stat3 enhances AR-mediated gene expression such as PSA.
- Stat3 enhances androgen independent growth of prostate cancer cells *in vitro* and *in vivo*.
- Stat3 activates androgen receptor (AR) in the presence and in the absence of androgen.

#### Reportable outcome

##### Manuscript:

DeMiguel F., Lou W., Lee S. O., Xiao X., Pflug B., Nelson J.B., and **Gao A.C.** Stat3 enhances the growth of LNCaP human prostate cancer cells in intact and castrated male nude mice. *The Prostate*, 52: 1-7, 2002.

## Conclusions

We demonstrated that activation of Stat3 in androgen-sensitive LNCaP prostate cancer cells results in enhancement of tumor growth in both intact and castrated male nude mice, and enhances androgen receptor-mediated prostate specific antigen (PSA) expression. These findings demonstrate that intracellular signaling mediated by Stat3 can enhance the growth of androgen-sensitive human LNCaP prostate cancer cells in both intact and castrated male nude mice.

## References

1. DeMiguel F., Lou W., Lee S. O., Xiao X., Pflug B., Nelson J.B., and **Gao A.C.** Stat3 enhances the growth of LNCaP human prostate cancer cells in intact and castrated male nude mice. *The Prostate*, 52: 1-7, 2002.
2. Lee S.O., Lou, W., Demiguel F, Hou, M., and Gao, A.C. Interleukin-6 promotes androgen-independent growth in LNCaP human prostate cancer cells. *Clin Cancer Res.* (in press)

## Stat3 Enhances the Growth of LNCaP Human Prostate Cancer Cells in Intact and Castrated Male Nude Mice

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**BACKGROUND.** Prostate cancer frequently progresses from an initial androgen dependence to androgen independence, rendering the only effective androgen ablation therapy useless. The mechanism underlying the androgen-independent progression is unknown. Stat3, a member of the family of signal transducers and activators of transcription, is activated in numerous cancers, including prostate. This study is to investigate the role of Stat3 activation in the growth of prostate cancer cells.

**METHODS.** A constitutively active Stat3 was ectopically expressed in androgen-sensitive LNCaP prostate cancer cells and resulting stable clones expressing activated Stat3 were isolated. The effect of Stat3 activation on LNCaP cell growth in response to androgen in vitro and in vivo was examined.

**RESULTS.** We show that the levels of activated Stat3 are associated with the progression of androgen-independent prostate cancer. Activation of Stat3 in androgen-sensitive LNCaP prostate cancer cells results in enhancement of tumor growth in both intact and castrated male nude mice and enhances androgen receptor-mediated prostate specific antigen expression.

**CONCLUSIONS.** These findings demonstrate that intracellular signaling mediated by Stat3 can enhance the growth of androgen-sensitive human LNCaP prostate cancer cells in both intact and castrated male nude mice. *Prostate* 52: 123–129, 2002. © 2002 Wiley-Liss, Inc.

**KEY WORDS:** Stat3; androgen-independence; androgen receptor; prostate cancer

### INTRODUCTION

Hormone-refractory prostate cancer refers to a resistance to androgen ablation therapy, the only effective systemic therapy available for advanced prostate cancer. Almost all patients with advanced prostate cancer respond initially to androgen ablation therapy. However, virtually every patient will relapse to hormone-refractory disease due to the growth of androgen-independent cancer cells. There is growing evidence supporting the concept that the paracrine and autocrine loops mediated by growth factors and cytokines play an important role in acquisition of hormone independence [1,2]. Stat3, a member of Janus Kinase (JAK)-Signal Transducers and Activators of Transcription (STAT) signaling pathway, is implicated in many cytokine-, hormone-, and growth factor-

mediated signaling pathways to regulate a variety of biological responses, including development, differentiation, cell proliferation, and survival [3,4].

Constitutively activated Stat3 protein is found in various types of tumors, including leukemia, breast,

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head and neck, and prostate [5–10]. In addition, constitutively activated Stat3 (a mutant produced by substitution of the cysteine residues within the COOH-terminal loop of the SH2 domain of Stat3) induces cellular transformation and tumor formation in nude mice [11]. These results suggest that Stat3 may function as an oncogene and play a critical role in transformation and tumor progression. Here, we investigated the effect of Stat3 activation on the growth of androgen-sensitive LNCaP cells in the intact and castrated male nude mice.

## MATERIALS AND METHODS

### Cell Culture and Plasmids

Human LNCaP prostate cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). The LNCaP cells were passaged twice weekly, and the passage number approximately eight were used for all of the studies. Androgen-insensitive LN95, LN96, LN97, and LN98 human prostate cancer cells [12] were maintained in phenol red-free RPMI 1640 supplemented with 10% charcoal-stripped serum (Hyclone, CA). HeLa cells were routinely maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% FBS. The cells were grown at 37°C in 5% CO<sub>2</sub> and 95% air. The plasmid (pSG5-wtAR) containing the wild-type full-length sequence of human androgen receptor (AR) was kindly provided by Dr. Chawnshang Chang, University of Rochester, NY. The plasmid containing the constitutively active form of Stat3c was provided by Dr. James Darnell, Jr., The Rockefeller University, NY [11]. Prostate specific antigen (PSA) regulatory element containing an 822-bp enhancer [13] and a 620-bp promoter [14] was generated by PCR amplification of Hirt DNA from LNCaP cells by using primers for the enhancer (5' primer, 5'-GCGGTACCCTGCAGAGAAT; and 3' primer, 5'-GGATCCCCATGGTTCTGTC), and for the promoter (5' primer, GGATCCTTGGATTTTGAAAT; and 3' primer, 5'-GGTCTAGAAAGCTTGGGGCT). The PCR products of the amplified enhancer and promoter were gel purified, kinased, and inserted into the *EcoRV* site of pBluescript KS+ vector, separately. The PSA regulatory element was generated by inserting the enhancer (cut out with *Bam*HI from the pBluescript KS+ construct) in front of the promoter at the *Bam*HI site of the pBluescript construct containing the promoter. The PSA reporter construct (pAAV-PSA-Luc) was generated by insertion of the 1.4-kb fragment of the PSA enhancer and promoter in place of the CMV promoter of the pAAV-CMV-Luc construct [15].

### Luciferase Assay

Twenty-four hours before transfection,  $3 \times 10^5$  cells were plated in a six-well plate in phenol red-free DMEM containing 5% dextran-coated charcoal-stripped FBS (CS-FBS). Cells were transfected with a total amount of 5 µg of DNA by using Superfect (Qiagen, Valencia, CA) according to the manufacturer's instructions. The total amount of plasmid DNA used was normalized to 5 µg/well by the addition of empty plasmid. Three hours later, the DNA:liposomes mixture was removed and cells were treated with phenol red-free medium containing 5% CS-FBS with either 10 nM dehydrotestosterone (DHT, Sigma, St. Louis, MO) or in the absence of DHT. Cell extracts were obtained 36 hr later, and luciferase activity was assayed by using the Luciferase Assay System (Promega, Madison, WI). Protein concentration in cell extracts was determined by Coomassie Plus protein assay (Pierce, Rockford, IL). Luciferase activities were normalized by protein concentrations of the samples. All transfection experiments were performed in triplicate wells and repeated at least four times.

### Transfection and Northern Blot

Transfections with the plasmid expressing the constitutively active form of Stat3c or empty vector were performed by using Superfect (Qiagen, Valencia, CA) according to the manufacturer's protocol. Stable clones were selected in 800 µg/ml G418 and maintained in 300 µg/ml G418.

Total RNA was extracted from cells with TRIzol reagent (Life Technologies, Rockville, MD). Twenty micrograms of each sample was electrophoresed in 1.2% denaturing agarose gels and transferred to a nylon membrane (MSI, Westborough, MA). A 1.2-kb *Bam*HI fragment of the PSA cDNA was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol, ICN, Costa Mesa, CA) by using the Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization was carried out during 3 hr at 65°C in Rapid-hyb buffer (Amersham). Membranes were washed for 15 min at 65°C in 2 × SSC, 0.1% sodium dodecyl sulfate (SDS; twice), 0.5 × SSC, 0.1% SDS and 0.1 × SSC, 0.1% SDS. Radioactivity in the membranes was analyzed with a Molecular Imager FX System (Bio-Rad, Hercules, CA).

### Electromobility Shift Assay

Whole cell extracts were prepared and electromobility shift assays (EMSAs) were performed as described previously [8]. For supershift analyses, the cell extracts were preincubated with antibody specifically

against Stat3 (Santa Cruz Biotechnology, Santa Cruz, CA). The protein-DNA complexes were resolved on a 5% nondenaturing polyacrylamide gel in  $1 \times$  TBE (90 mM Tris-borate, 2 mM EDTA) at room temperature, and the results were analyzed as above.

#### Western Blot

Whole cell extracts were obtained by lysing the cells in RIPA buffer ( $1 \times$  phosphate buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, with freshly added protease inhibitors: 0.1 mM phenylmethyl sulfonyl fluoride, 1 mM sodium orthovanadate, 30  $\mu$ l/ml aprotinin). Whole cell extracts were resolved in 12.5% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membrane. After blocking overnight at  $4^\circ\text{C}$  in 5% milk in PBS-0.1% Tween 20, membranes were incubated for 1 hr at room temperature with anti-AR rabbit polyclonal antibody (Santa Cruz Biotechnology) or anti-FLAG antibody (Sigma), or anti-phospho-Stat3 antibody (Cell Signaling Technology, MA) diluted in 1% milk in PBS-Tween. After secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England).

#### In Vivo Assays

The mice were injected in the flank with  $3 \times 10^6$  cells resuspended in Matrigel diluted 1:1 in complete culture medium. The volume of the growing tumors was estimated by measuring their three dimensions (length  $\times$  width  $\times$  depth) with a caliper.

#### PSA Protein Analysis

PSA secretion was quantitated by PSA immunoradiometric assay (Beckman Coulter, Fullerton, CA) of tissue culture supernatant. Equal numbers of cells were plated in phenol red-free RPMI containing 10% FBS. Cells were allowed to attach for 24 hr, then the medium was changed to phenol red-free medium supplemented with 10% charcoal-stripped serum. After another 2 days, 50  $\mu$ l of supernatant was assayed for PSA.

#### Statistical Analysis

Values were expressed as the mean  $\pm$  SE. Statistical analyses were performed by one-way analysis of variance, followed by the Student-Newman-Keuls test for multiple comparisons, with a  $P < 0.05$  being considered significant.

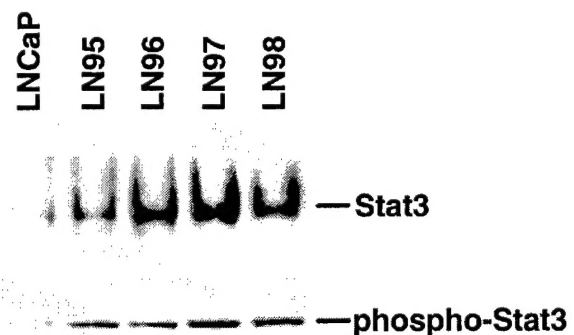
## RESULTS AND DISCUSSION

### Stat3 Activation Is Associated With Androgen-Independent Progression

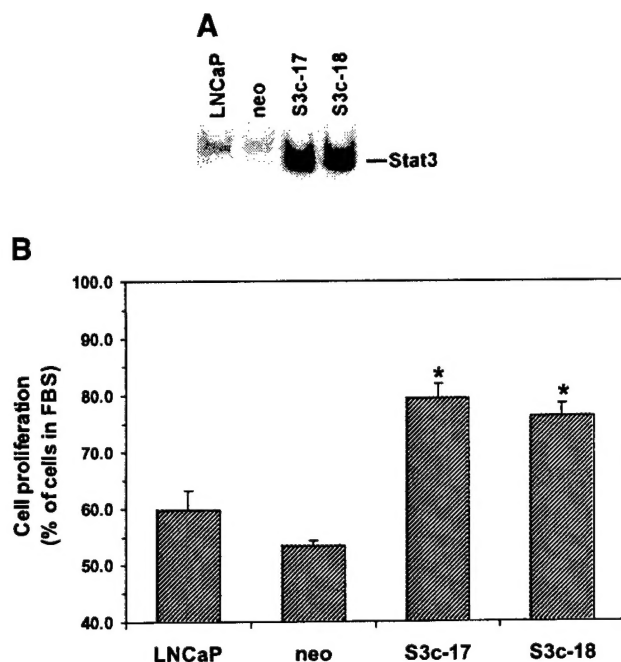
We first tested whether Stat3 activation is associated with the progression of androgen-independent prostate cancer. We analyzed Stat3 activity by EMSA and the expression of phosphorylated Stat3 protein in androgen-sensitive LNCaP human prostate cancer cells and androgen-insensitive sublines (LN95, LN96, LN97, and LN98) derived from LNCaP cells [12]. LNCaP sublines (LN95, LN96, LN97, and LN98) were derived from LNCaP cells after chronic androgen deprivation in vitro, which produces androgen-insensitive clones [12]. All of the LNCaP sublines retain the AR and produce PSA even in the absence of androgen [12]. The androgen-insensitive LNCaP sublines grew readily in both castrated and intact male nude mice compared with no tumors formed in wild-type LNCaP animals subcutaneously [12]. Both the levels of Stat3 activity and phosphorylated Stat3 protein were increased in the androgen-insensitive sublines compared with that of the parental LNCaP cell line (Fig. 1). These results suggest that androgen-insensitive growth is associated with increased levels of Stat3 activity in LNCaP human prostate cancer model.

### Stat3 Activation Enhances LNCaP Cell Growth In Vitro and In Vivo

Because LNCaP cells have minimal levels of Stat3 activity, we tried to determine whether elevation of



**Fig. 1.** Stat3 activation is associated with androgen-independence. **Top panel:** Stat3 DNA-binding activity in LNCaP and LN series cell lines. Whole cell extracts (20  $\mu$ g) were subjected to EMSA by using a  $^{32}\text{P}$ -labeled oligonucleotide probe containing the consensus binding motif for Stat3. **Bottom panel:** Stat3 tyrosine phosphorylation (phospho-Stat3) in LNCaP and LN series cell lines. Forty micrograms of whole cell protein extract was analyzed by Western blot by using antibodies specific against phosphotyrosine Stat3 (Tyr-705) antibody.



**Fig. 2.** Stat3 enhances androgen-independent growth in vitro. **A:** Stat3 DNA binding activity in Stat3-overexpressing clones (S3c-17, S3c-18), vector control (neo), and LNCaP cells examined by EMSA. **B:** Effect of Stat3 on LNCaP cell growth in the presence and absence of androgen in vitro. Cells were cultured in RPMI-1640 supplemented with either 10% FBS or 10% charcoal-stripped FBS. Cell proliferation values in charcoal-stripped FBS were expressed as percentage relative to the complete FBS. \* $P < 0.05$ .

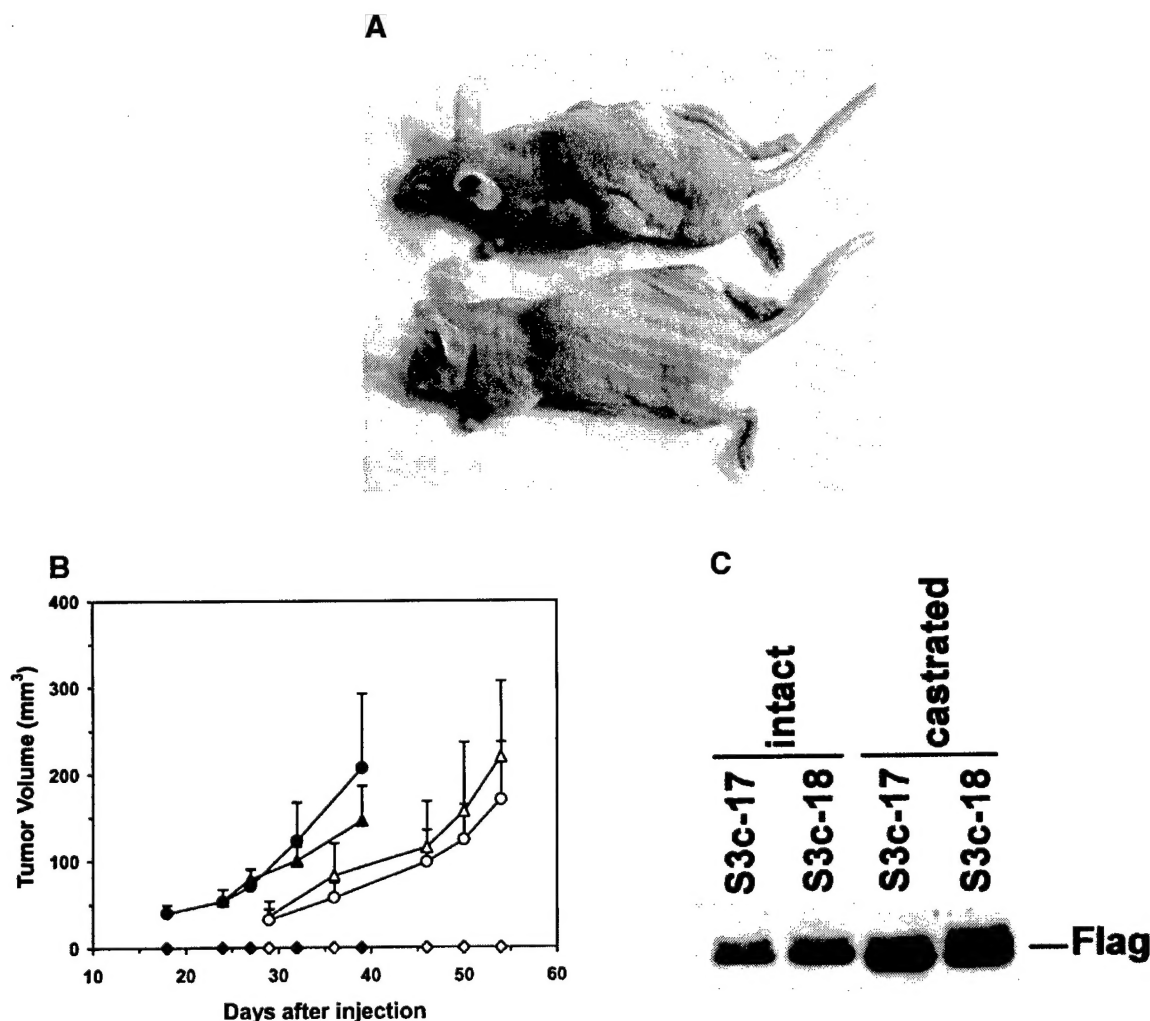
Stat3 activity will enhance LNCaP cell growth in vitro and in vivo. We introduced a constitutively activated Stat3 into the androgen-sensitive LNCaP cells. Two independent clones overexpressing activated Stat3 were selected (Fig. 2A). The growth of these cells in normal serum and in androgen-deprived serum was compared. The growth of LNCaP cells and neo vector alone control cells in culture was reduced by approximately 50% after 48 hr in androgen-deprived charcoal-stripped serum compared with that in the normal serum (Fig. 2B). In both clones of LNCaP cells overexpressing Stat3, however, there was only a 20% decrease in growth under these androgen-deprived conditions compared with growth in normal serum (Fig. 2B), suggesting that activated Stat3 can enhance the growth of LNCaP cells in the absence of androgen in vitro.

LNCaP is a human prostate cancer cell line derived from supraclavicular lymph node metastases [16,17]. These cells express mutant but functional androgen receptors and exhibit androgen-sensitive phenotype

[16,17]. Igawa et al. reported that the aggressiveness and androgen responsiveness of LNCaP cells can be altered by culturing the cells continuously in vitro [18]. LNCaP cells in the early passages are usually less aggressive (i.e., lower tumorigenic in vivo) and higher androgen responsiveness in vitro and in vivo than that of the cells in their late passages [18]. The parental LNCaP cells used in the present study are in their early passages (see Materials and Methods section). The parental LNCaP cells and vector control Neo clone did not grow any detectable tumor in both intact (within 40-day observation period) and castrated (within 60-day observation period) male mice (Fig. 3B). We next tested the effect of Stat3 activation on LNCaP tumor growth in both intact and castrated male nude mice. Eight-week-old male nude mice were randomly divided into two groups, one left intact, another group received surgical castration. Three days after castration, intact or castrated male nude mice were injected subcutaneously with LNCaP cell clones overexpressing Stat3. For the two independent Stat3-overexpressing clones, tumors became apparent at the site of injection within 20 days in the intact male mice and within 30 days in the castrated male mice (Fig. 3A). There was a delay of the latency for tumor formation in the castrated male mice compared with the intact male mice (Fig. 3B). Western blot analysis of protein extracts derived from Stat3-overexpressing tumors in both intact and castrated male mice revealed high levels of FLAG-tagged Stat3 protein (Fig. 3C), indicating that the growing tumor cells continue to express FLAG-tagged Stat3 plasmids and are derived from human LNCaP cells. These results demonstrate that Stat3 activation not only enhances prostate cancer cell tumor growth in vivo, but also promotes tumor growth in the androgen-deprived castrated male nude mice.

#### Stat3 Enhances AR-Mediated Gene Expression Independent of Androgen

To determine whether Stat3 activation affects AR signaling, we tested the effects of Stat3 on the expression of endogenous PSA, a well-characterized prostate specific antigen whose transcription is strictly regulated by androgen [19]. The levels of PSA mRNA expression from LNCaP cell clones overexpressing activated Stat3 were increased compared with the parental LNCaP cells (Fig. 4A). To examine the effects of Stat3 activation on the expression of PSA upon androgen withdrawal, we measured PSA protein secretion in phenol red-free medium supplemented with the charcoal-treated serum. As shown in Figure 4B, the levels of PSA protein expression were increased in the Stat3-overexpressing LNCaP

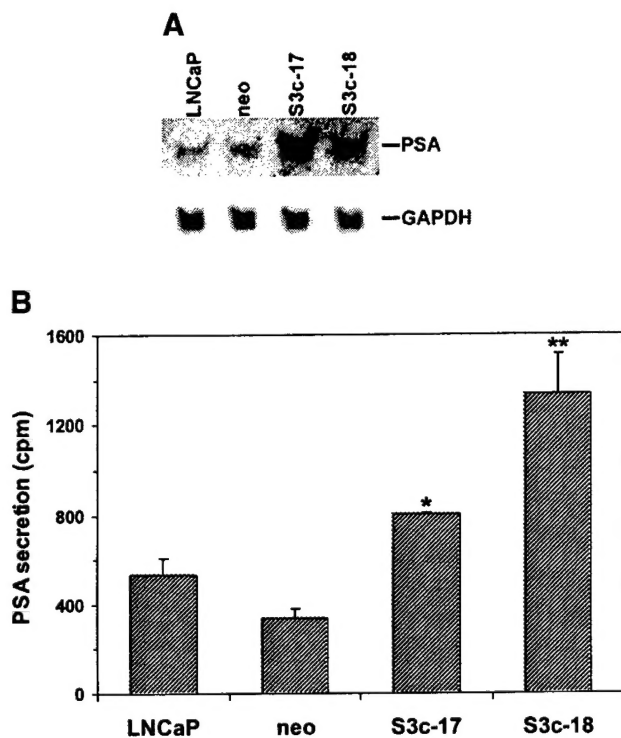


**Fig. 3.** Stat3 induces androgen-independent growth in vivo. **A:** Stat3-overexpressing clone (S3c-18) developed tumors vs. parental LNCaP cells, which did not grow any tumors in the castrated male nude mice. **B:** Tumor growth curve in the intact and castrated male nude mice. Parental LNCaP cells and neo clone ( $\diamond$ ) or clones that overexpress activated Stat3 (S3c-17, triangles; S3c-18, circles) were injected into the intact (filled symbols) or castrated (open symbols) male nude mice ( $n = 10$  for each condition). **C:** Western blot analysis of Flag-tag expression in cell extracts from Stat3-derived tumors in the intact and castrated male nude mice.

subclones compared with the parental LNCaP cells and vector controls in androgen-deprived conditions, indicating that Stat3 can partially replace androgen function in activation of the AR-mediated PSA gene expression. Tumors expressing Stat3 also produced high levels of circulating PSA in the serum (average, 32 ng/ml per gram of tumor) in the castrated male mice.

To determine the effect of Stat3 activation on AR-mediated gene transcription, we transiently transfected LNCaP cells with a luciferase reporter linked to the androgen-responsive promoter of PSA and

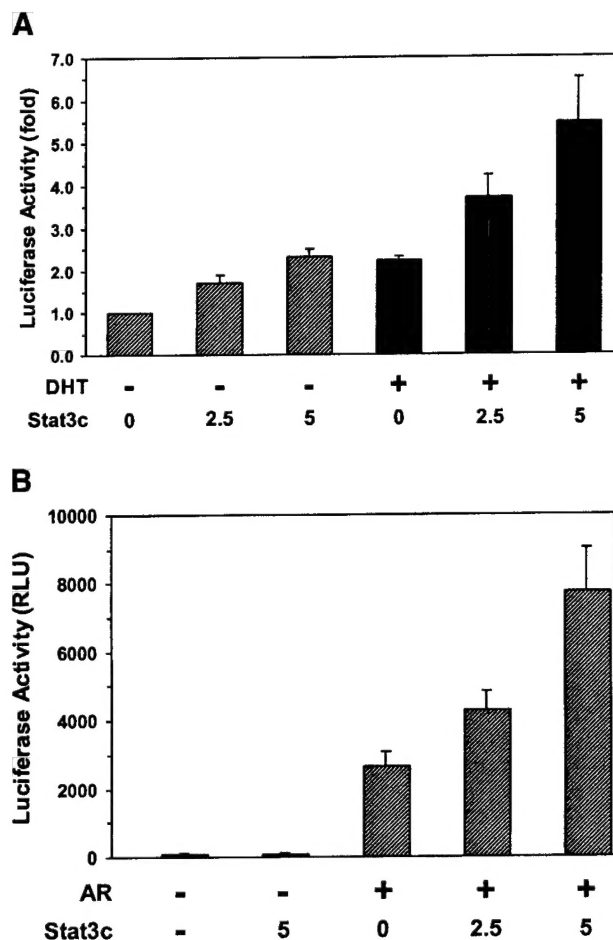
various amounts of expression vectors encoding the constitutively active Stat3 [11]. To compare the effect of Stat3 on PSA promoter activity in the presence and in the absence of the androgen, the cells were then cultured in phenol red-free medium supplemented with the charcoal-stripped serum either in the presence of 10 nM of dehydrotestosterone (DHT) or in the absence of DHT. After 24 hr, cells were harvested and luciferase activities were determined. As shown in Figure 5A, Stat3 activated the PSA-luc reporter in a concentration-dependent manner in the absence of androgen, suggesting that Stat3 activates PSA tran-



**Fig. 4.** Stat3 enhances prostate specific antigen (PSA) expression. **A:** PSA mRNA expression in Stat3-overexpressing clones (S3c-17, S3c-18), vector control (neo), and LNCaP cells examined by Northern blot analysis by using 20  $\mu$ g of total RNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a control for equal loading. **B:** PSA protein secretion in the absence of androgen. PSA secretion was quantitated by PSA immunoradiometric assay of 50  $\mu$ l of supernatant of cell culture in phenol red-free RPMI containing 10% charcoal-stripped serum. \* $P < 0.05$ ; \*\* $P < 0.01$ .

scription in a ligand-independent manner. Addition of 10 nM of DHT-enhanced PSA-luc reporter activity induced by Stat3 (Fig. 5A). To test whether the effects of Stat3 on PSA transcription require AR, we transiently transfected AR-negative HeLa cells [20], with or without an AR expression vector, plus a luciferase reporter with the androgen-responsive promoter of PSA (PSA-Luc), and increasing amounts of expression vector encoding the constitutively active Stat3c. Cotransfections lacking the AR failed to result in activation of PSA-luc reporter gene (Fig. 5B), suggesting that the effects of Stat3 on androgen responsive gene expression are dependent on AR.

Our findings demonstrate that increased Stat3 activity promotes tumor growth of androgen-sensitive LNCaP prostate cancer cells in both intact and castrated male nude mice and enhances AR-mediated



**Fig. 5.** **A:** Effect of Stat3 on prostate specific antigen (PSA) promoter activity in the absence of dehydrotestosterone (DHT) and in the presence of 10 nM of DHT. LNCaP cells were transiently transfected with PSA-luc reporter and increasing doses (0, 2.5, 5  $\mu$ g) of Stat3 expression plasmid. Total DNA content was kept constant in all wells. **B:** The effect of Stat3 on PSA transcription requires androgen receptor (AR). HeLa cells transiently transfected with or without AR expression plasmid, PSA-luc reporter, and increasing doses (0, 2.5, 5  $\mu$ g) of Stat3c expression plasmid in the presence of 10 nM of DHT. Total DNA content was kept constant in all wells. The luciferase activity was measured. Results are displayed as the average of four independent experiments. RLU, relative light units.

PSA expression both in the presence and absence of androgen.

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